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Phylogeographic Analysis of Pigtail Macaque Populations (*Macaca nemestrina*) Inferred From Mitochondrial DNA

LISA L. ROSENBLUM,[1]* JATNA SUPRIATNA,[2]
AND DON J. MELNICK[1,3,4]
[1]*Genetics Laboratory, Department of Anthropology, Columbia University, New York, New York 10027*
[2]*Department of Biological Sciences, University of Indonesia, Depok, Indonesia*
[3]*Department of Biological Sciences, Columbia University, New York, New York 10027*
[4]*Center for Environmental Research and Conservation, Columbia University, New York, New York 10027*

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ABSTRACT Mitochondrial DNA variation was surveyed in nine populations of the pigtail macaque (*Macaca nemestrina*), covering all three recognized subspecies in Southeast Asia. To do this, a 2,300 base pair fragment spanning the mitochondrial NAD 3 and NAD 4 genes and flanking tRNA subunits leucine and glycine was targeted for amplification and digested with a battery of 16 restriction endonucleases. Out of a total of 107 individuals, 32 unique haplotypes could be distinguished. Parsimony and neighbor-joining analyses grouped the haplotypes into five strongly supported assemblages representing China/Thailand, Malaysia, Sumatra, Borneo, and Siberut. These results indicate that the mainland and island mtDNA haplotypes are strictly and uniquely limited to the geographic ranges of the recognized morphological subspecies. Cladistic and neighbor-joining analyses indicate that inferred phylogenies of mtDNA haplotypes are congruent with subspecies designations. Furthermore, in support of morphological studies, results indicate that the Mentawai macaque is most likely not a distinct species but a subspecies of *M. nemestrina*. Am J Phys Anthropol 104:35–45, 1997.

Because of its unique mode of inheritance and rapid evolution in some genes, mitochondrial DNA (mtDNA) has been used extensively to study intraspecific population structure and its close association with historical geographic events (Riddle and Honeycutt, 1990; Bernatchez and Dodson, 1991; Allard and Honeycutt, 1992; Avise et al., 1992; McMillan and Palumbi, 1995). Closely related populations that become geographically isolated may experience an interruption in gene flow. Eventually, evolutionarily independent units, which can be identified by morphological and genotypic differences, are formed. Other, completely unrelated species with similar geographic distributions may have concordant patterns of population and phylogenetic structures simply due to shared histories of geographic events. These shared patterns can be used to infer fundamental historical biogeographic processes (Avise et al., 1987; Morrone and Crisci, 1995).

Phylogenetic patterns of colonization and extinction associated with the complex geological history of the Indonesian archipelago

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Correspondence to: Lisa L. Rosenblum, Jefferiss Trust Laboratories, Floor 2, St. Mary's Hospital Medical School, Praed Street, London W2 1NY, England. E-mail: L.Rosenblum@ic.ac.uk

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have received much attention (Groves, 1985; Heaney, 1986). However, little is known about within-species genetic variability in the fauna of the region. As pigtail macaques (*Macaca nemestrina*) have a broad geographic distribution that covers much of the Southeast Asian mainland and islands (Fig. 1), they provide an excellent opportunity to study genetic variation associated with the evolutionary and geological history of Southeast Asia and the Sunda shelf. The Sunda shelf refers to the continental shelf of Southeast Asia, an area that is now covered by shallow waters but is believed to have been exposed for a substantial period of time (20,000 years) leading up to and following the last glacial maximum (approximately 18,000 YBP). The islands that lie on the shelf that were connected during this period include Borneo, Sumatra, and Java and a host of smaller islands.

Three subspecies of pigtail macaques have been recognized on the basis of morphological characters, such as pelage color, pattern, and tail morphology (Fooden, 1975). *M. n. leonina* occupies southern mainland Southeast Asia extending south to the Khlong Marui Fault in the Malay Peninsula (Fig. 1). *M. n. nemestrina* is found in southern peninsular Malaysia, Sumatra, and Borneo (Fig. 1). *M. n. pagensis*, the Mentawai macaque, recognized by some as a distinct species (Tenaza, 1975; Wilson and Wilson, 1976), is restricted to the Mentawai islands, off the west coast of Sumatra (Fig. 1). Thus, populations of pigtail macaques are isolated by distance, where larger genetic differentiation is expected over greater distances in a nonfragmented habitat (Wright, 1969) and time, where genetic differentiation depends partly on population size and genetic variability of the founder population (Nozawa et al., 1977; Nei, 1978).

These biogeographical parameters provide an excellent opportunity to examine the distribution of populations and correlates of phylogenetic diversity in *M. nemestrina*. A phylogeny of mtDNA haplotypes was established from individuals representing populations over much of the geographic range of the three subspecies of *M. nemestrina* to address the following questions. First, are the phylogenetic patterns derived from mtDNA haplotypes in *M. nemestrina* congruent with known historical biogeographical events in Southeast Asia and the Sunda shelf? Second, are morphologically distinguishable subspecies discrete phylogenetic units that can be recognized in the phylogenetic analysis of their mtDNA? Third, is the Mentawai macaque a distinct species?

MATERIALS AND METHODS

DNA collection and preparation

Blood samples were obtained from *Macaca nemestrina* individuals throughout the range of all three subspecies (Fig. 1; Table 1). Individuals in Sumatra were sampled on the east and west sides of the Pegunungan Barisan mountain range and a relatively isolated population in the northwestern-most part of the sampling range near Lake Mannangau. Samples were collected as 5 ml of whole blood in a syringe and diluted with an equal volume of 100 mM Tris, 100 mM EDTA, and 2% SDS in the field (Rassman et al., 1989). The blood samples were stored at ambient temperature until they could be digested with proteinase K and total DNA extracted once with phenol/chloroform [1:1(vol./vol.)] and once with chloroform (Dowling et al., 1990). DNA was recovered by centrifugal dialysis in Centricon-30 columns (Amicon, Beverly, MA) and resuspended with a solution of 1 mM Tris-HCL, 0.1 mM EDTA, pH 7.5 (Kocher et al., 1989).

Amplification

The 2,300 base pair (bp) mitochondrially encoded NAD dehydrogenase complex, subunits 3 and 4 (Chomyn et al., 1985) and flanking tRNA regions were specifically targeted for polymerase chain reaction (PCR) (Saiki et al., 1987) by a universal oligonuleotide primer pair. The primers are located in the glycine region adjacent to NADH 3 (5′-TAAC/TTAGTACAGC/TTGACTTCCAA-3′) and leucine flanking NADH 4 (5′-TTTTG-GTTCCTAAGACCAAC/TGGAT-3′) (Cronin et al., 1993).

A sample of DNA was subjected to 30 cycles of amplification in a 100 µl reaction volume using standard PCR protocols (Saiki et al., 1987). PCR reactions contained 1.5 units of Taq DNA polymerase (Perkin Elmer-

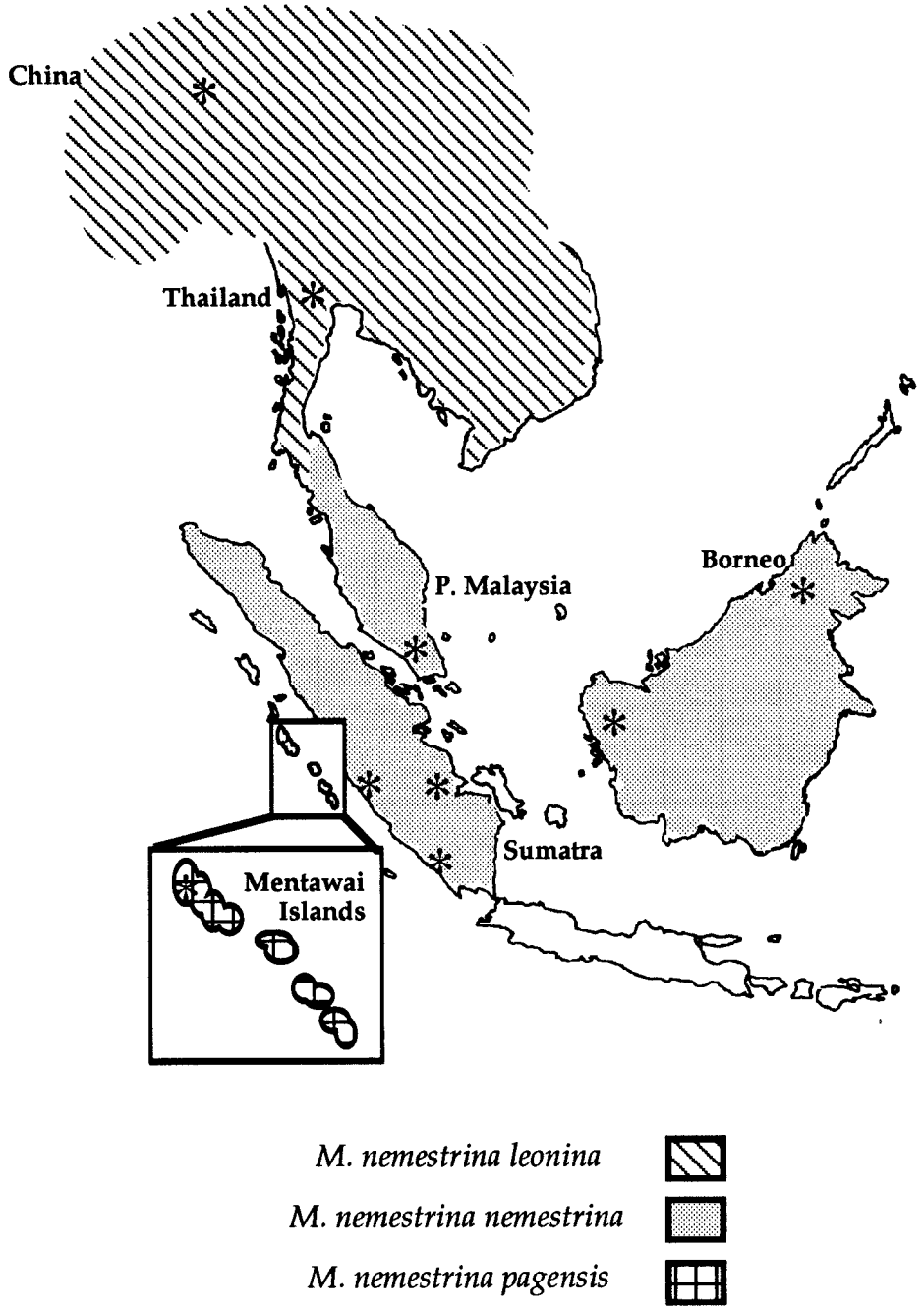

Fig. 1. Map of pigtail macaque distribution and locations of sample populations used in this study. The three subspecies are represented by different hatching patterns.

Cetus, Norwalk, CT), 20 pm/µl of each primer, 25 mM $MgCl_2$, each dNTP at 2 mM, 1 mg/ml bovine serum albumin, 500 mM KCl, and 100 mM Tris-HCL buffer, pH 8.3, to form double-stranded DNA of the entire 2,300 bp fragment. Cycle parameters were set for 45 s at 95°C for denaturation, 40 s at 53°C for annealing, and 2 min at 72°C for

TABLE 1. List of Macaca nemestrina *populations and mtDNA haplotypes*[1]

Subspecies and region	Population location	mtDNA number	Individuals
M. n. leonina			
China	Kunming Institute of Zoology, SW China	M0 (2)	2[2]
Thailand	Dusit Zoo, Bangkok	M1 (2)	2[2]
M. n. nemestrina			
Malaysia	Melaka Zoo, S. Peninsula	M2 (1), M3 (1), M4 (4), M5 (1), M6 (2), M7 (2)	11[2]
Sumatra	Sumatera Selatan	I3 (1), I4 (25), I5 (1), I6 (1), I7 (2), I8 (1), I9 (2), I17 (8), I18 (1)	42
	Sumatera Barat	I9 (5), I10 (5), I11 (2), I12 (1), I13 (1), I15 (2), I16 (6)	22
	Lake Mannangau	I15 (3), I16 (11), I19 (1), I14 (1)	16
Borneo	Sabah	I1 (1), I2 (1)	2
	Sarawak	I0 (1)	1
M. n. pagensis			
Mentawai	Siberut	I20 (1), I21 (4), I22 (3), I23 (1)	9[2]

[1] The mtDNA haplotypes found, frequencies and total number of individuals analyzed from each population are given.
[2] Zoo-captive, wild-born individuals.

extension. Negative and positive controls were used during each PCR set. Amplified products were visualized along with a known size standard ladder on ethidium bromide–stained gels under UV light.

Restriction site mapping

Individuals were first screened for unique haplotypes by complete digestion of the amplified segment (Dowling et al., 1990). Five microliters of amplified DNA from each of the 107 macaques was digested with an excess of each of the following 16 restriction enzymes: *Bsm* I, and *Dra* I, which recognize unambiguous six base sequences; *Acc* I, *Bbs* I, and *Bsp* 1286 I, which recognize ambiguous six base sequences; *Bbv* I, *BsmA* I, *Bsr* I, and *Hph* I, which recognize unambiguous five base sequences; *Sau* 96 I, which recognizes ambiguous five base sequences; *BstU* I, *Dpn* I, *Hae* III, *Hha* I, *Hpa* II, and *Nla*, III all of which recognize unambiguous four base sequences. These enzymes were specifically chosen to minimize recognition sequence overlap and thus avoid overestimates of sequence divergence (see Hugall et al., 1994). Restriction digests were performed following the manufacturer's instructions (New England BioLabs, Beverly, MA). Digested DNA fragments were separated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, and photographed with Polaroid film under UV light. Fragments were sized against a 1 kb ladder standard run on each gel.

For restriction site mapping, DNA of selected individuals was again amplified with the same primer pair. For this amplification step, the heavy strand primer was modified to include a biotin molecule on its 5′ end. Amplification was followed by partial cleavage with a particular restriction enzyme (see Morales et al., 1993). This procedure was repeated for all 16 endonucleases. The resulting fragments were run alongside two biotinylated known size standards (New England BioLabs), øX174 DNA digested with *Hae* III, and Lambda DNA digested with *BstE* II, and separated by electrophoresis on 1.5% agarose gels. DNA fragments were transferred to a Nylon membrane (GIBCO-BRL, Gaithersburg, MD) by standard blotting procedures, fixed to the membrane by UV light, and detected by chemiluminescent chemicals (Photogene Nucleic Acid Detection System; GIBCO-BRL). Membranes were exposed to x-ray film for 1–5 h. Site positions were measured from the labeled primer end and compared directly to the known size standards in the autoradiogram. This procedure has been successfully applied in a wide array of taxa (Morales and Melnick, 1994; Morales and Bickham, 1995).

Statistical analysis of sequence polymorphisms

Restriction sites for each individual from all 16 restriction endonucleases were scored directly from autoradiograms, assembled in a presence-absence matrix (available on re-

quest), and analyzed by the REAP computer package (McElroy et al., 1992). Population structure was analyzed by an analysis of molecular variance (AMOVA) (Excoffier et al., 1992). A permutational approach was used to test the variance components and phi statistics.

Each unique pigtail macaque haplotype was operationally treated as a taxon and collectively analyzed using a heuristic search with random addition options in PAUP, version 3.1.1 (Swofford, 1993) (Fig. 2). Characters were weighted Dollo-up (LeQuesne, 1974; Farris, 1977), and support for major nodes on the inferred tree is shown as the percentage agreement in a consensus of trees produced from 1,000 bootstrap simulations of the data set (Penny and Hendy, 1985). Trees were also constructed using the neighbor-joining method (Saitou and Nei, 1987) from pairwise distance matrices estimated using Nei and Li's model (1979) (Fig. 3). Both inferred trees are unrooted.

RESULTS

Phylogenetic analysis of mtDNA haplotypes

A total of 107 individuals from nine populations in all three *M. nemestrina* subspecies were sampled (Table 1). For each macaque, a 2,300 base pair fragment was digested with 16 endonucleases yielding 80 variable characters, 75 of which were parsimony informative. Thirty-two haplotypes could be distinguished based on the 75 variable positions among these haplotypes. The binary matrix of variable positions and corresponding haplotypes is available on request. For convenience, *M. nemestrina* haplotypes from islands were designated I0 through I23, and haplotypes found on the mainland (China, Thailand, and peninsular Malaysia) were designated M0 through M7 (Table 1).

The 32 haplotypes were operationally treated as taxa and analyzed using parsimony by a heuristic search in PAUP (Swofford, 1993) and by neighbor-joining to estimate branch lengths (Saitou and Nei, 1987). A 50% majority rule consensus tree, 74 steps long, was inferred from parsimony analysis (Fig. 2). There was strong support for five major clades comprising two mainland assemblages, Thailand/China and Malaysia, and three island assemblages including Sumatra, Borneo, and Siberut. Haplotypes within the major assemblages, however, were not well resolved. The major phylogenetic relationships within the pigtail macaques inferred from neighbor-joining analysis were in agreement with parsimony analysis (Fig. 3).

Results from the analysis of molecular variance (AMOVA) reflected the well-defined clades inferred from parsimony and neighbor-joining analyses. Hierarchical analysis of mtDNA variability showed substantial subdivision among macaque populations (Table 2). While most of the variation was found among regions (>63%), an appreciable amount was also found among populations within regions (>25%). Relatively little variation was distributed among individuals within populations. Two locations in Borneo and three in Sumatra were sampled and defined as populations. The two populations in Borneo were well differentiated in both parsimony and neighbor-joining analyses (Figs. 2, 3). However, the populations in Sumatra were not well resolved in either analysis (Figs. 2, 3) in spite of the significant population structuring ($P < .0001$) indicated by AMOVA (Table 2).

Geographic distribution of mtDNA haplotypes

The distribution of haplotypes within the pigtail macaques was found to be highly correlated with geography (Fig. 3; Table 2). No haplotype was shared among regions, and branch lengths from neighbor-joining analysis indicated large distances among them (Fig. 3). The geographic structure of haplotype distribution was least pronounced among individuals from Thailand and China (Figs. 2, 3). Despite the fact that the geographic distribution of pigtail macaques is continuous from China through Thailand and Malaysia, members of different subspecies were separated by a substantial branch length of .05494 between the Thailand/China assemblage and the Malaysia haplotype cluster (Figs. 1, 3).

Haplotype structure was less resolved by parsimony analysis among populations in Sumatra (Fig. 2). Of the 17 haplotypes found in Sumatra, nine were associated with Su-

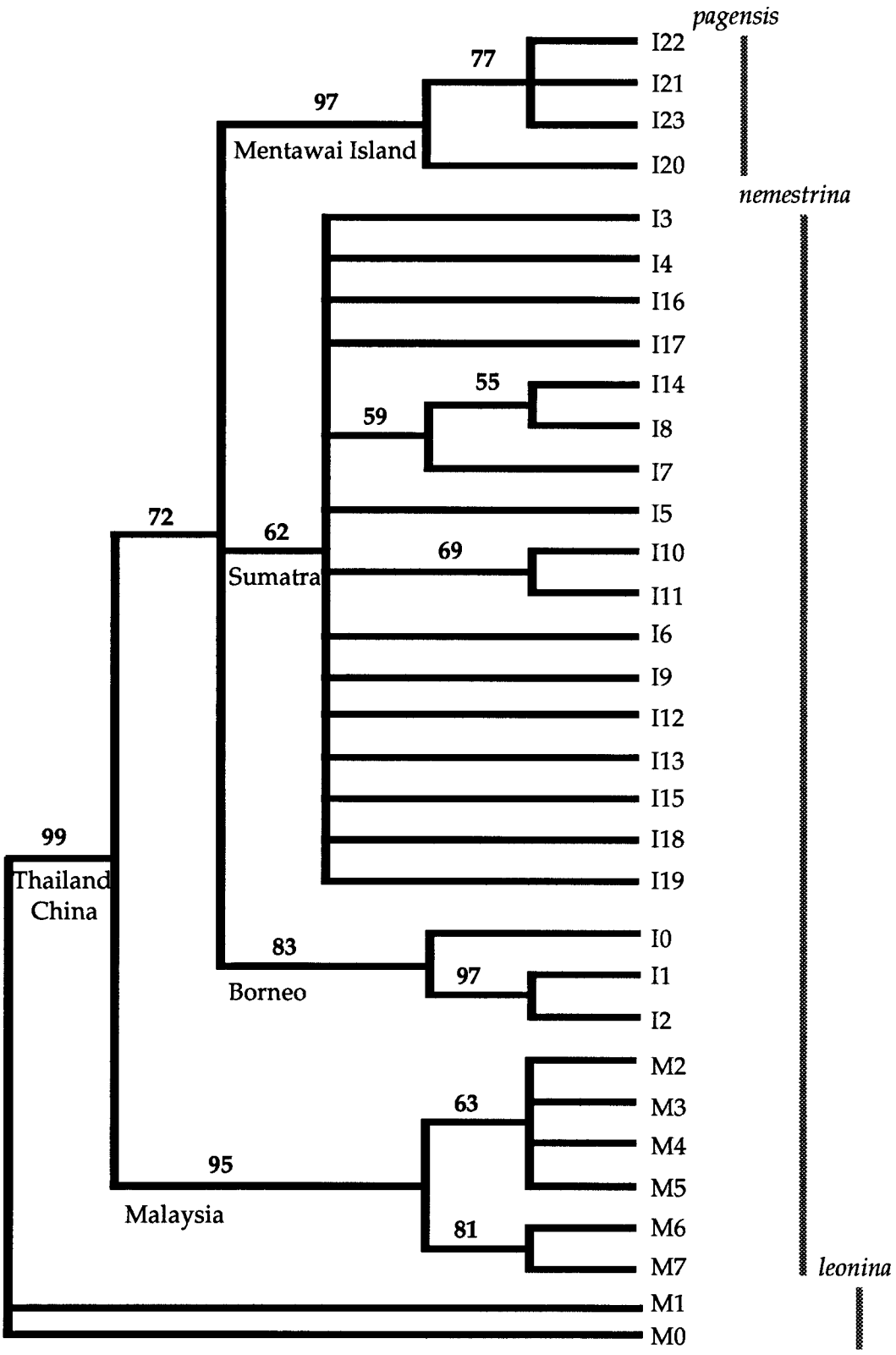
pagensis
I22
77
I21
97
I23
Mentawai Island
I20
nemestrina
I3
I4
I16
I17
55
I14
59
I8
I7
I5
72
62
69
I10
Sumatra
I11
I6
I9
I12
I13
I15
99
I18
Thailand
China
I19
I0
83
97
I1
Borneo
I2
M2
63
M3
M4
95
M5
Malaysia
81
M6
M7
leonina
M1
M0

Figure 2.

matera Selatan (east of the mountain range), seven with Sumatera Barat (west of the mountain range), and four with the Lake Mannangau area, the northernmost and isolated sampling area west of the range (Fig. 1; Table 1). Thus, three haplotypes were shared among populations: I15, I16, and I9 (Table 1). Haplotypes I15 and I16 were found in Lake Mannangau and Sumatera Barat, both west of the mountain range. Haplotype I9 was found in two individuals east of the range and five west of it. The two individuals originated from the same sampling location east of the range. That a single haplotype out of 17 was found on both sides of the mountain range indicates some level of geographic population structure, even if it could not be well resolved in parsimony analysis.

On the other hand, the distribution of three haplotypes found in two locations in Borneo were well differentiated in neighbor-joining (Fig. 3) as well as resolved with strong support in parsimony analysis (Fig. 2).

The geographic structuring of mtDNA haplotypes reflected the distribution of morphologically recognized subspecies: haplotypes M0 and M1 were confined to *M. n. leonina;* M2 through M7 as well as I0 through I19 were confined to *M. n. nemestrina;* I20 through I23 were only found in *M. n. pagensis* (Table 1; Fig. 2). The range of *M. n. leonina* is limited to China and Thailand and *M. n. nemestrina* to peninsular Malaysia, Sumatra, and Borneo. Despite the continuous distribution of *M. n. leonina* and *M. n. nemestrina* found in peninsular Malaysia, neighbor-joining analysis revealed a substantial distance between the two haplotype clusters (Fig. 3). *M. n. nemestrina* was highly differentiated among all regions examined, indicating a close association of population divergence with historical geographic events.

Fig. 2. Phylogenetic hypothesis for the relationship among 32 haplotypes found in *M. nemestrina* constructed using parsimony analysis. Because of the large number of taxa, a subset of possible trees was evaluated by a random addition heuristic search with 1,000 bootstrap replications. The cladogram represents an unrooted 50% consensus tree. Support for major nodes within the tree is shown as the percentage agreement in the consensus tree. Haplotypes designated M0 through M7 are found on mainland Southeast Asia. Haplotypes designated I0 through I23 are found on offshore islands.

Similarly, neighbor-joining analysis indicated that *M. n. pagensis*, whose range is limited to the Mentawai Islands, was genetically distant from all other geographic assemblages (Fig. 3). *M. n. pagensis* was genetically closer to some populations of *M. n. nemestrina* than to others. To summarize the findings, the mainland and island mtDNA haplotypes are strictly and uniquely limited to the geographic ranges of the morphological subspecies.

DISCUSSION

Relationships among populations and their geographic distributions

The analysis presented here suggests that the current geographic distribution of haplotypes is likely the result of two main processes during the evolution of pigtail macaque mtDNA. First, global biogeographic processes resulted in a historically early separation between what are now separate subspecies. Second, there has been a more recent differentiation of lineages within each of the major groups, defined by parsimony and neighbor-joining analyses as China/Thailand, Malaysia, Borneo, Sumatra, and Mentawai Islands (Figs. 2, 3). This more recent lineage separation is particularly indicated by the fact that the relationship of the haplotypes from three locations in Sumatra was not well resolved, nor did they correspond well to current geography in the cladistic and neighbor-joining analyses (Fig. 2). The exceptions are haplotypes from Borneo which represent a geographically differentiated, highly supported clade.

The current distribution of divergent haplotypes is associated with geographical processes in Southeast Asia during the Pleistocene. The shape and duration of various land bridges have been inferred using sea-level curve data (Milliman and Emery, 1968; Emery et al., 1971). Because the Sunda Shelf is covered by very shallow water, it appears that mainland Southeast Asia, Java, Borneo, and Sumatra were last connected between 27,000 to 7,000 BP in the Pleistocene to form the extinct subcontinent of Sundaland. Islands in the Mentawai archipelago are thought to have had a peninsular connection to Sumatra and thus the rest of the Asian mainland during the maximum

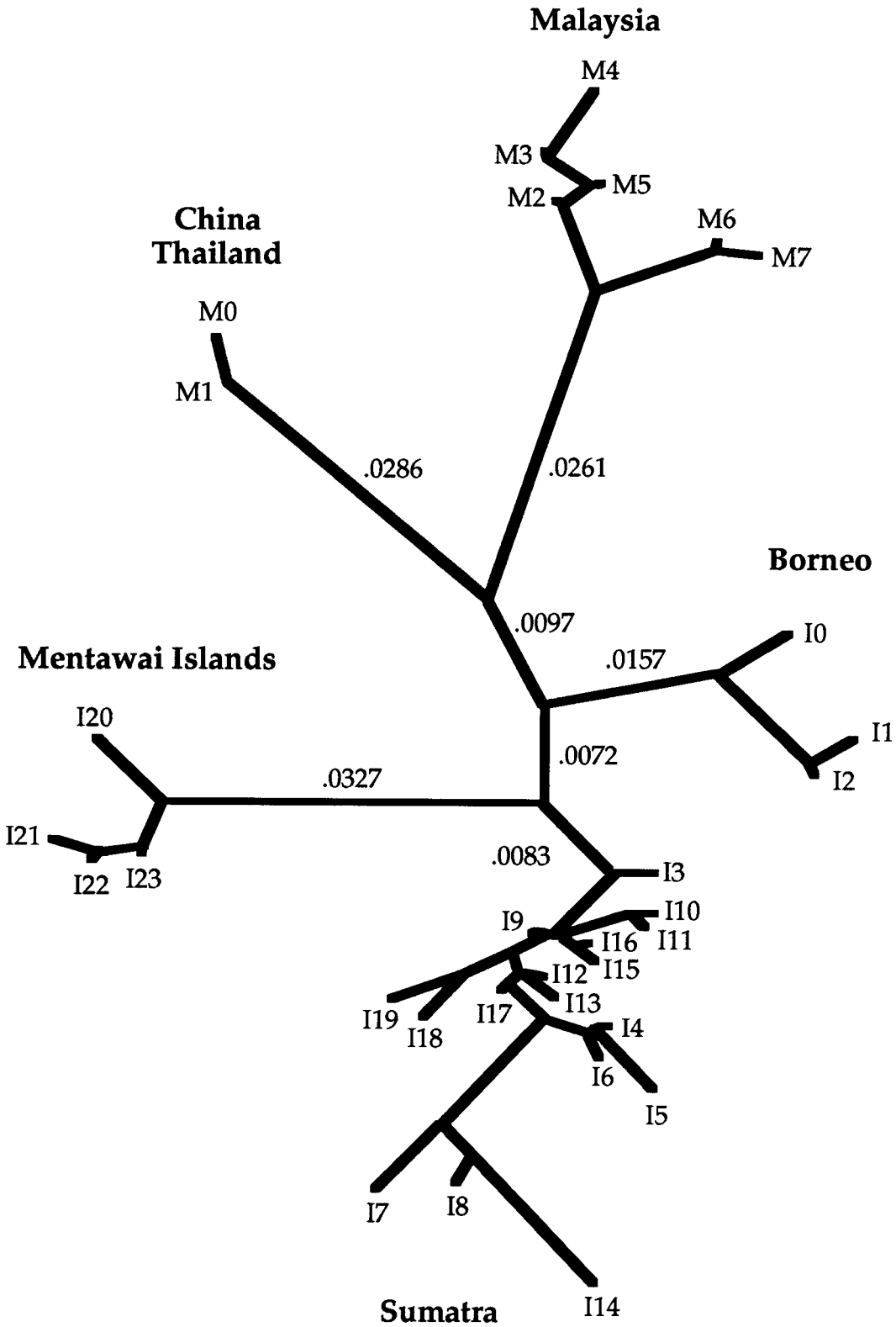

Fig. 3. Network of haplotypes within *M. nemestrina* constructed by the neighbor-joining method. Branch lengths for the five major assemblages are noted.

TABLE 2. Hierarchical analysis of variance between haplotypes[1]

Variance component	Variance	% total	*P*[2]	Phi statistics
Among regions	4.844	63.13	.0046	.621
Among populations	2.017	25.87	.0001	.683
Within populations	.936	12.01	.0001	.880

[1] Regions and populations for AMOVA analysis are defined as given in Table 1.
[2] Probability of having a more extreme variance component and phi statistic than observed values by chance alone.

sea level lowering of 200 m, which occurred in the middle Pleistocene between 160,000 and 180,000 BP (Heaney, 1986). Paleofaunal and paleobotanical evidence suggests that these land connections allowed large mammals, including primates, to expand their ranges and colonize both the Greater Sunda islands and the nearby Mentawai archipelago (Audley-Charles, 1987). Therefore, macaques could have migrated over this continuous land bridge until around 7000 BP.

Subspecies designation and mtDNA

If populations, such as the pigtail macaques, are separated for relatively long periods of time by biogeographic barriers, then accumulated differences in the mtDNA would also be recognized. What is known of the pigtail macaque migration and subsequent subspeciation has been summarized by Fooden (1975, 1980). The early pigtail stock dispersed south to peninsular Malaysia and the Sunda shelf. The pigtail stock reached the Mentawai Islands, west of Sumatra, facilitated by a land bridge connection that formed during maximum sea-level lowering that occurred around 180,000 BP. The separation and subsequent morphological divergence of *M. n. nemestrina* and *M. n. leonina* may have been caused by a marine transgression that bisected the Thai-Malay peninsula at the Khlong Marai Fault. The well-defined clades of *M. n. leonina* found in Thailand and China and of *M. n. nemestrina* in Malaysia reflect this earlier biogeographical divergence of two subspecies whose distribution is not now separated by water (Fig. 3). Haplotypes associated with *M. n. pagensis* are unique and quite divergent from the other two subspecies, thus supporting the morphological subspecies designation which is discussed in detail below (Fig. 2). The extreme geographic structuring of haplotypes within *M. n. nemestrina* can be attributed to biogeographical separation of these populations by water followed by accumulated mutations in the mtDNA genome. We also suggest that extreme female philopatry in macaques contributes to the observed population differentiation, accelerating the accumulation of genetic differences by impeding the flow of mtDNA even during periods of contact (Melnick and Hoelzer, 1992, 1993).

M. n. pagensis or *M. pagensis?*

The taxonomic placement of the Mentawai macaque, *M. n. pagensis*, has generated considerable controversy worth discussing in the context of this study. *M. n. pagensis* is recognized by Fooden (1975) as a subspecies of *M. nemestrina* based on cranial and tail morphology and coat color. Others consider the Mentawai macaque to be a distinct species whose close relative is *M. fascicularis*, not *M. nemestrina* (Tenaza, 1975; Wilson and Wilson, 1976). The conclusion from these studies that the Mentawai macaque is a distinct species is based mostly on the biogeographical inference that the Mentawai macaque was isolated in the early Pleistocene from the main ancestral stock. The results from mtDNA analysis in this study are clearly in agreement with Fooden's (1975) morphological analysis resulting in the designation of the Mentawai macaque as a subspecies. Neighbor-joining analysis showed genetic distances between haplotypes in the Mentawai macaque and those in other subspecies to be mostly within the range of distances between haplotypes of *M. n. nemestrina* and *M. n. leonina* (Fig. 3). The distance between haplotypes of *M. n. leonina* in China and those of *M. n. nemestrina* in peninsular Malaysia is 0.0547 (Fig. 3). Similarly, the pairwise distances between the Mentawai macaque and *M. n. nemestrina* in peninsular Malaysia, *M. n. nemestrina* in Borneo, and *M. n. nemestrina* in Sumatra are 0.0685, 0.0556, and 0.041, respectively (Fig. 3). The largest genetic distance of 0.0782 lies between haplotypes of the Mentawai macaque and *M. n. leonina* (Fig. 3). A more complete analysis requires fur-

ther sampling of Mentawai macaque individuals from the Pagai islands in order to make a stronger case for subspecies status based on mtDNA analysis. However, a study in which samples from the Pagai Islands were included has estimated similar genetic distances from the entire mtDNA genome, further supporting a subspecific designation (Williams, 1990).

CONCLUSIONS

AMOVA analysis of mtDNA variability revealed substantial subdivision among pigtail macaque populations, with most variation found among regions. In contrast, little variation was distributed among individuals in the same region. Additionally, the distribution of haplotypes within the pigtail macaques was found to be highly correlated with geography. These results indicate that biogeographical separation of these populations by water was followed by accumulated mutations in the mtDNA genome. We suggest that gene flow was further impeded by extreme female sedentism found in pigtail and other macaque monkeys. Genetic differences were allowed to accumulate in the mtDNA molecule, contributing to the observed dramatic geographic structuring of maternally inherited genetic variation.

Neighbor-joining analysis revealed that biogeographic processes resulted in a historically early separation between what are now separate subspecies. The geographic structuring of mtDNA haplotypes reflects the distribution of the three morphologically recognized subspecies. Results inferred from this study strongly support conclusions from morphological studies on *Macaca nemestrina* subspeciation. Finally, the genetic distances between *M. n. pagensis* and the other *M. nemestrina* subspecies, coupled with results from the cladistic analysis, indicate that the Mentawai macaque is not a separate species. Thus, the results from this study are clearly in agreement with Fooden's (1975) morphological analysis resulting in the designation of the Mentawai macaque as a subspecies of pigtail macaque.

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